Report 2182

RAPID BIOASSAY FOR WATER SOLUBLE FUNGITOXICANTS

June 1976

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: 2182 (14) USAMERDC-2182
2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER

4. TITLE *(and Subtitle)*: (6) RAPID BIOASSAY FOR WATER SOLUBLE FUNGITOXICANTS
5. TYPE OF REPORT & PERIOD COVERED: Final; July 1971 through December 1975
6. PERFORMING ORG. REPORT NUMBER
(9) Final Rpt. July 1971-Dec 1975

7. AUTHOR(s): (10) Gertrud E. Ernst
8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS: U.S. Army Mobility Equipment Research and Development Command ATTN: DRXFB-V Fort Belvoir, Virginia 22060
10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: PRON: AW-6-R0003-01-AW-EF; AMS No.: 612105.11H8400; Proj No.: 1T162105AH84

11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Mobility Equipment Research and Development Command, Fort Belvoir, Virginia 22060
12. REPORT DATE: June 1976
13. NUMBER OF PAGES: 19 (12) 14 pp

14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*
15. SECURITY CLASS. *(of this report)*: Unclassified
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*
(16) DA-1-T-162105-AH-84 PROJN-AW-6-R0003-01-AW-EF

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17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*
Bioassay
Candida albicans
Fungitoxicants
Germ Tubes
Media

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A screening method for water soluble fungitoxicants was developed which gives results within a workday. The test method is based on the faculty of *Candida albicans* to form germination tubes within 3 to 6 hours' incubation in certain growth promoting media.

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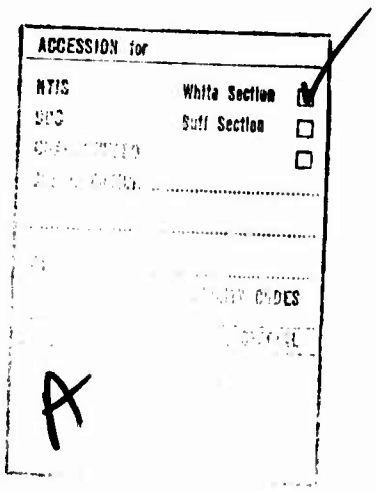
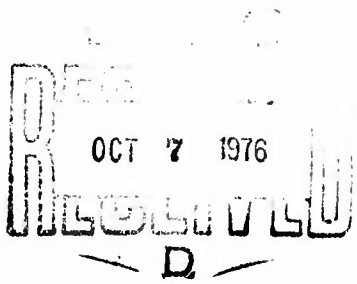
SUMMARY

A simple bioassay method for water soluble fungicides using *Candida albicans*, a yeastlike organism, as test organism and human serum,[1] plasma, suitable other organic or synthetic products, and commercially prepared horse serum or other animal sera,[2] as media in which *C. albicans* form germ tubes, is described. *C. albicans* is added to different concentrations of a fungicide in a growth stimulating medium and incubated for 3 hours at 34° C. After completion of the incubation time, 100 cells are counted under the microscope, and the number of cells having germ tubes is noted. A control treated in the same manner but without addition of the fungicide is observed with every test.





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2 E. R. Griffin, 1964, "The Value of the Germ Tube Production Test in the Rapid Identification of *Candida albicans*," J. of Med. Lab Tech. *21:* pp. 298-301.

PREFACE

The work was accomplished by Gertrud E. Ernst under the direction of Emil J. York, Chief, Laboratory 9000, MERADCOM. The manuscript was revised and advice was given by Sidney Levine, Chief, Chemistry Research Group. Able technical assistance was rendered by Victoria L. Manzano and Kathleen A. Yauss, summer students.

CONTENTS

RAPID BIOASSAY FOR WATER SOLUBLE FUNGITOXICANTS

I. INTRODUCTION

1. Statement of the Problem. The goal was to develop a simple bioassay for which the ingredients are readily available and with which test results can be obtained within a workday.

2. Background. Fungal growth is considered a primary cause of biodeterioration of materials which are not rendered fungus resistant or are not inherently resistant to fungus growth. Since the growth of fungi and their metabolic activities are accelerated in the humid, hot climate of the tropics and subtropics, where much of the Army's materiel is stored and utilized, fungicides are widely used by the Army to protect the materiel against biodeterioration. Rapid and simple screening methods for fungicides are, therefore, of importance.

II. EXPERIMENTAL PROCEDURE

3. Approach to the Problem. Laboratory experiments were carried out with four chlorophenolic compounds of Dow Chemical Company, Midland, Michigan, and their effects on the germination of three different strains of *C. albicans*[3] were established. In order to evaluate the minimal effective amount of the fungitoxicants, they were tested in serial twofold dilution bioassays using *C. albicans* as test organism in a suitable growth promoting medium. In initial tests, the germ tube formation was observed after 2, 3, 4, 5, and 6 hours of incubation at 34° C. It was established that the germ tube formation in human plasma at 34° C was optimal after 3 hours ± ½ hour. The tests were read routinely, therefore, after 3 hours' incubation.

III. RESULTS

4. Results. The following chlorophenolic compounds (Table 1) supplied by the Dow Chemical Company, Midland, Michigan, were used as test fungicides:

[3] *C. albicans* 3476, 3477, and 3478 were obtained from the U.S. Army Regional Medical Laboratory, Fort George Meade, Odenton, Maryland.

Table 1. Composition of Test Fungicides

Name	Components	Composition (%)
Dowicide A	Active-Sodium o-Phenylphenate–$4H_2O$	97
	Inert Ingredients	3
Dowicide B	Active-Sodium Trichlorophenate	85
	Inert Ingredients	15
Dowicide F	Active-Sodium Pentachlorophenate	79
	Sodium salts of other chlorophenols	11
	Inert Ingredients	10
Dowicide G	Active-Sodium Tetrachlorophenate	80
	Inert Ingredients	20

The Dowicide was dissolved in water or saline solution on a weight/volume basis.

The following ranges (Table 2) were tested:

Table 2. Range of Fungicide Concentration

Fungicide	Concentration Range (%)	Minimum Effective Amount (%)
Dowicide A	0.4–0.006	0.4
Dowicide B	0.2–0.009	0.15
Dowicide F	2.0–0.004	0.25
Dowicide G	2.0–0.016	0.125

A slight sensitivity variation to the same fungicide was noticed in different strains. Variations in test results were also present if too many *C. albicans* cells were added. In the above tests, human plasma was used which was derived from an expired human blood transfusion unit, Group B, Rh+. Experiments showed that the age of the plasma did not influence the test results provided that the plasma was not deteriorated. *C. albicans* readily form germ tubes in bovine (calf) serum,[4] which can be substituted, therefore, for human serum or plasma. In an attempt to find a commercially obtainable medium, dehydrated skim milk (Difco) was reconstituted, inoculated with *C. albicans*, and incubated; but, it proved not suitable as a medium for the assay because only rare germ tubes were formed. Albumen from fresh eggs was considered as

[4] Bovine serum is available from several meat product companies such as Armour.

a possible substitute medium and was tested both undiluted and diluted with physiological saline (0.9 percent) in 1:1 and 1:2 ratios and incubated for 3 and 6 hours at 34° C. The undiluted albumen promoted 83 percent germ tube formation after 3 hours' incubation and almost 100 percent after 6 hours. In the diluted albumen, the percentage of germ tube formation depended greatly on the dilution factor and was very low at the necessary dilution. The difficulty in pipetting fresh undiluted albumen and the fact that the addition of the aqueous fungicide dilutes the albumen to an extent that its ability to form germ tubes is greatly diminished makes it unsuitable for the test. Further experiments were carried out with reconstituted Beef Blood Serum (Difco). Only 28 percent of *C. albicans* cells germinated after 3 hours' incubation, but good results were achieved after enriching the medium with 0.025 percent yeast extract (Difco) reconstituted in modified Sabouraud dextrose solution[5] (see Table 3).

IV. DISCUSSION

5. Discussion. The twofold serial dilution technique is a satisfactory method to evaluate water soluble fungicides and to determine the minimum amount necessary to inhibit the formation of germ tubes in *C. albicans*. The bioassay described above uses human plasma derived from an expired blood transfusion unit; however, calf or beef serum can be substituted. Several commercial media were tried for the serial twofold dilution. The best results were obtained with a growth promoting medium utilizing reconsitttuted, dried beef blood serum with added yeast extract reconstituted in modified Sabouraud's medium.

Synergistic or antagonistic combinations of fungitoxic substances can be determined by mixing the stock solutions of the fungitoxicants before diluting.

The short test duration made it unnecessary to sterilize glassware or media before use or to apply aseptic technique. After test completion, however, it was necessary to sterilize all glassware and media contaminated with *C. albicans* to avoid possible laboratory infection with the test organism.

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[5] Formula in grams per liter of distilled water:

Peptone	10
Dextrose	40
Yeast extract	25
Final pH	5.6

Table 3. Germ Tube Toxicity Test Results; Twofold Dilution Method (Phase I and II Combined)

Strain No.	Dowicide	Control	Concentration (%)*																			
			2.0	1.0	0.5	0.4	0.25	0.2	0.15	0.125	0.1	0.075	0.062	0.05	0.038	0.031	0.025	0.019	0.016	0.012	0.009	0.007	0.006	0.004
3476	G	+++	0	0	0																			
3477	G	+++	0	0	0																			
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3476	F	+++		0	0		0			+			+			++			+++			+++		+++
3477	F	+++		0	0		0			0			+			++			+++			+++		+++
3478	F	+++		0	0		0			+			++			+++			+++			+++		+++

Note:

Test organism: *Candida albicans*
Plasma: Human blood (expired), Group B, Rh+
Control: 0.9 cm^3 plasma; 0.1 cm^3 organism suspension in physiological saline
Readings taken after 3 hours at 34° C

*Key:

No germ tubes: 0
Occasional tubes: +
Many tubes: ++
Tubes equal to control: +++

V. CONCLUSIONS

6. Conclusions. It is possible to screen water soluble fungicides in the time frame of one workday. The described method is simple, fast, and reproducible. Human plasma was found to be the best suited medium because of the great number of germ tubes formed within 3 hours of incubation and because of its other properties: no disturbing particle suspension, easy to pipette, and long shelf life when refrigerated. If human plasma is not available, however, other media may be substituted with satisfactory results.

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APPENDIX

BIOASSAY PROCEDURE FOR WATER SOLUBLE FUNGITOXICANTS

1. Equipment and Materials.

a. Apparatus:

(1) Analytical balance (Mettler)
(2) Two beam balance
(3) Microscope
(4) Autoclave
(5) Incubator
(6) Serological pipettes, 1 ml, divided in 1/100 graduations
(7) Measuring pipettes, 10 ml, divided in 1/10 graduations
(8) Test tubes 13 x 100 mm (Wasserman tubes)
(9) Erlenmeyer flasks, 500 and 1,000 ml
(10) Microscope glass slides and cover slips
(11) Bunsen burner
(12) Inoculating loop
(13) Petri dishes

b. Solutions, chemicals, and media:

(1) NaCl
(2) Mycosel agar (B.B.L.)
(3) Skim milk, dehydrated
(4) Beef Blood Serum, dehydrated
(5) Yeast extract, dehydrated

2. Procedure.

a. The fungitoxicant to be tested was weighed on the Mettler balance, dissolved, and diluted to the desired concentration.

b. A suspension in physiological saline (0.9 percent NaCl) of a 48-hour-old culture of *C. albicans* on Mycosel agar (B.B.L.) was prepared. Physiological saline solution was used because of its isotonic properties.

c. A twofold serial dilution was set up. (See Table 4.)

Table 4. Method for Preparing Serial Twofold Dilution

Tube	Volume (ml)						
	Control	1	2	3	4	5	6
Medium	0.5	0.9	0.5	0.5	0.5	0.5	0.5
Fungicide Stock Solution	—	0.1	0.5 of tube 1	0.5 of tube 2	0.5 of tube 3	0.5 of tube 4	0.5 of tube 5[a]
Final concentration[b]							
C. albicans in saline	0.05	0.05	0.05	0.05	0.05	0.05	0.05

[a] Discard 0.5 ml

[b] Depending on stock-solution concentration

3. **Method for Preparing Twofold Serial Dilution.** Place the required number of test tubes (13 x 100 mm), one for each fungicide dilution and one for the control, in a test tube rack. To the first tube, add 0.9 ml of medium; to each of the following tubes, add 0.5 ml of medium (plasma or as desired). To the first tube, add 0.1 ml of fungicide stock solution. Mix well, transfer 0.5 ml to the next test tube, mix well, and proceed as with the first tube. Continue to the last tube and discard the last 0.5 ml. A control tube containing 0.5 ml of medium only is set up with every test. Add to every test tube 0.05 ml (or 1 drop) of the test organism suspension. Mix well. Incubate at 34° C for 3 hours. Mix well and place one drop on a glass slide. Count the germ tubes formed in 100 *C. albicans* cells present under low microscopic magnification (500X magnification) to obtain the percentage of germ tubes formed. The lowest concentration of fungicide which suppresses the germ tube formation is the effective amount. The serial dilution test was carried out in two phases. First, a wide range was tested to determine the range of fungitoxic activity. Then, the dilution row was started with an amount slightly higher than the endpoint amount and carried down to a minimal necessary amount of fungitoxicant in order to obtain a more accurate range.

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